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Abstract—It was the purpose of the present study to evaluate glutamate-stimulated phosphatidylinositol metabolism in primary mixed astrocyte/neuron and neuron-enriched cortical cultures through different stages of development *in vitro*. Glutamate (0–200  $\mu$ M) stimulated inositol phosphate accumulation in a concentration-dependent fashion at 6, 13 and 20 days *in vitro*. Pure astrocyte cultures exhibited glutamate-stimulated phosphatidylinositol hydrolysis only at high concentrations (100–400  $\mu$ M), indicating that these cells contribute little to the overall inositol phosphate accumulation measured in mixed neuronal cultures treated with low glutamate concentrations. Comparison of mixed neuronal cultures with and without antimetabolic treatment revealed that increasing astrocyte number suppressed glutamate-stimulated responses, presumably via glutamate uptake. In contrast to previous reports, glutamate-stimulated inositol phosphate accumulation, when expressed as a function of cell number, increased with increasing days *in vitro*.

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DEVELOPMENT OF GLUTAMATE-STIMULATED  
PHOSPHATIDYLINOSITOL METABOLISM IN PRIMARY NEURONAL  
AND ASTROCYTE CULTURES

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**Abstract**—It was the purpose of the present study to evaluate glutamate-stimulated phosphatidylinositol metabolism in primary mixed astrocyte/neuron and neuron-enriched cortical cultures through different stages of development *in vitro*. Glutamate (0–200  $\mu$ M) stimulated inositol phosphate accumulation in a concentration-dependent fashion at 6, 13 and 20 days *in vitro*. Pure astrocyte cultures exhibited glutamate-stimulated phosphatidylinositol hydrolysis only at high concentrations (100–400  $\mu$ M), indicating that these cells contribute little to the overall inositol phosphate accumulation measured in mixed neuronal cultures treated with low glutamate concentrations. Comparison of mixed neuronal cultures with and without antimetabolic treatment revealed that increasing astrocyte number suppressed glutamate-stimulated responses, presumably via glutamate uptake. In contrast to previous reports, glutamate-stimulated inositol phosphate accumulation, when expressed as a function of cell number, increased with increasing days *in vitro*.

**Key words:** glutamate, inositol phosphates, development, primary cortical cultures, neurons, astrocytes.

Expression of the metabotropic glutamate (Glu) receptor (mGluR1) linked to phosphatidylinositol bis-phosphate (PI) metabolism generally increases in the central nervous system of neonatal to 5-week-old rats.<sup>8</sup> Furthermore, a comparison of the distribution of this Glu receptor subtype between developing and adult rat brain indicates that this increase is in accordance with the maturation of glutamatergic neuronal elements.<sup>17</sup> However, development of activity of the mGluR1 does not appear to undergo parallel regulation with receptor expression.<sup>3,10,11</sup> mGluR1 responses are greatest early in development, before and during the process of synaptogenesis *in vivo*.<sup>3</sup> Thereafter, Glu-stimulated inositol phosphate (IP) formation appears to diminish progressively to negligible levels around the 24th day of life in rats.<sup>10</sup>

In most studies evaluating development of excitatory amino acid (EAA)-stimulated PI metabolism, the effect of changing populations of neurons and astrocytes has not been considered. It is now known that EAA receptors (including metabotropic) are found on both of these cell types.<sup>7</sup> Astrocytes had been regarded only as uptake sites for EAAs before the observation of Glu-stimulated PI metabolism in pure cultures of these cells.<sup>13</sup> In the present study, we sought to evaluate the contribution of proliferating astrocytes on the development of EAA-stimulated PI metabolism in mixed cultures. By treating cultures with cytosine arabinoside (AraC) to control astrocyte proliferation, Glu-stimulated PI metabolism was compared in neuron-enriched and mixed cultures at 6, 13 and 20 days *in vitro* (DIV) and in essentially pure astrocyte cultures. By determining neuron, astrocyte and total cell numbers in these cultures, we have addressed the developmental dynamics of Glu-induced PI hydrolysis.

## EXPERIMENTAL PROCEDURES

*Primary cortical cultures*

Primary cultures were established using cerebral cortices from embryonic day 15 rats, as described previously.<sup>4</sup> Brain tissue was removed from each embryo, taking care to isolate and

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**Abbreviations:** AraC, cytosine arabinoside; DIV, days in vitro; EAA, excitatory amino acid; EDTA, ethylenediaminetetraacetate; Glu, glutamate; IP, inositol phosphate; mGluR1, metabotropic glutamate receptors; NCM, neuronal culture medium.

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discard meninges and blood vessels (thus removing the major source of fibroblasts and microglia). A single cell suspension was obtained by physical trituration of the pooled brain tissue in neuronal culture medium (NCM). Viable cell number was determined using Trypan Blue and a hemacytometer. Cells were plated at a density of 500,000 cells per well on 48-well plates coated with poly-L-lysine. During exchanges of medium and subsequent experiments, cells remained on the original plates.

Neurons were cultured in NCM containing 10% fetal calf serum and 10% horse serum. In some cultures (+AraC), 4 days after plating (4 DIV), cells were treated with  $1 \times 10^{-5}$  M AraC. Sister cultures (-AraC) were treated with the same volume of NCM rather than NCM with AraC. Four days later (8 DIV) on all plates, medium was removed from cells and replaced with minimal essential medium plus 10% horse serum. There were no other exchanges of medium or supplements made throughout the culturing period. Cells were used for experiments after 6, 13 or 20 DIV; therefore, cells at 6 DIV had been on AraC (or not) for 2 days, cells at 13 DIV had been off AraC (or not) for 5 days, and cells at 20 DIV off (or not) for 12 days.

#### *Secondary astrocyte cultures*

Cells obtained as above were plated on flasks, without AraC treatment, and were washed with NCM plus serum three times at 4, 9 and 15 DIV. This process depletes cultures of neurons, as described previously.<sup>2,15</sup> Astrocytes were removed from flasks using trypsin/EDTA and plated on 48-well plates at a density of 500,000 cells per well as described above. Cells were allowed to attach for 5 days before experimentation. Using this paradigm, cultures were determined to be approximately 95 % astrocytes as indicated by immunocytochemical markers (see below).

#### *Immunocytochemistry*

The immunocytochemical markers glial fibrillary acidic protein, neuron-specific enolase and myelin basic protein were used to identify astrocytes, neurons and oligodendrocytes, respectively, in mixed neuronal and pure astrocyte cultures. Primary antibodies raised in rabbit (DAKOPATTS, Dako Corp., Carpinteria, CA) were diluted 1:20 from each prediluted kit. Non-immune rabbit serum was used as the negative control. Alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, MO) with naphthol AS Mx phosphate and Fast True Red (Sigma) were used to generate the specific reaction precipitate. The supernatants were removed and absorbance measured at 510 nm as an indication of reaction product. Photomicrographs were also taken to identify the presence of specific cell types under differing culture conditions.

#### *Inositol phosphate accumulation*

The medium of cell cultures was replaced with freshly gassed (95% O<sub>2</sub>-5% CO<sub>2</sub> Krebs-Henseleit bicarbonate buffer. Measurement of PI metabolism was performed as described earlier,<sup>21</sup> except that cell cultures were placed in a 5% CO<sub>2</sub> incubator at 37°C for labeling with [<sup>3</sup>H]inositol and agonist stimulation. IP accumulation in the presence of LiCl in cells was terminated with 10% trichloroacetic acid. The cells were trituated and washed from the wells and total IP eluted from AG-1 X8 (formate type) anion exchange columns with 5 ml of 0.1 M formic acid/1.0 M ammonium formate.

#### *Cell counts*

Determination of total cell count was automated with the use of the Insight laser scanning confocal microscope (Insight system, Meridian Instruments, Inc., Okemos, MI). Twelve wells from each plate were treated as described above but [<sup>3</sup>H]-inositol was not added and, at the point of termination of IP accumulation, these wells were fixed and stained with Diff-Quik stain (Baxter Health Care Corp., McGaw Park, IL). This stain set is a modification of the Wright stain technique. The anionic dye component stains RNA while the cationic dyes stain DNA.<sup>20</sup> This process results in differential staining of astrocytes and neurons, which was evaluated by digitizing three fields in each well under white light using the Insight System (X40 objective, 220×190 μm area scanned per field). Digitized images were analyzed (IQ software, Meridian Instruments) by converting stain uptake to a linear grey scale. Overlapping cells were digitally separated and were sorted on the basis of staining intensity with neurons averaging two-fold the staining intensity of astrocytes. The

resultant biphasic distributions of cell populations vs staining intensity allowed for estimation of astrocyte/neuron cell number. All automated cell counts were confirmed by manual counts using the same dye. No significant difference was found between astrocyte, neuron or total cell number obtained from the two different counting methods.

## RESULTS

Figure 1 illustrates the Glu concentration-dependent stimulation of IP accumulation in secondary astrocyte cultures at 20 DIV, and in primary cortical cultures at 6, 13 and 20 DIV. The estimated  $EC_{50}$  values for maximal Glu stimulation of IP accumulation were  $24 \pm 1.2$ ,  $9 \pm 1.7$  and  $3 \pm 2.9$   $\mu$ M at 6, 13 and 20 DIV, respectively, using a graded dose-response procedure.<sup>19</sup> Maximum stimulation occurred at 100  $\mu$ M for 6 DIV and 40–50  $\mu$ M at 13 and 20 DIV. Maximal levels of stimulation were approximately 900% for 6 DIV and 500% for both 13 and 20 DIV. Pure astrocyte cultures at 20 DIV exhibited only 228% stimulation at 400  $\mu$ M Glu.

To address possible changes in culture composition with time *in vitro*, we investigated Glu-stimulated IP accumulation normalized to total cell number at 6, 13 and 20 DIV for paired cultures treated with or without AraC. As shown in Fig. 2 (6 DIV), AraC treatment for 2 days results in a higher plateau of IP accumulation/cell compared to cultures not treated with AraC. Although the ratio of neurons to astrocytes is altered by AraC treatment, total cell number is not. However, the shape of the response curves is similar for the +AraC and -AraC cultures.

By 13 DIV, total cell number is halved in +AraC cultures and has doubled in -AraC cultures (Fig. 3) as compared to 6 DIV (Fig. 2). In -AraC cultures at 13 DIV, there is a larger number of astrocytes in comparison to 6 DIV. Since both the neuronal and astrocyte numbers have been reduced between 6 (Fig. 2) and 13 DIV (Fig. 3) in +AraC cultures, this treatment has reduced astrocyte proliferation and neuronal viability. This result is consistent with the findings of Martin and colleagues,<sup>6</sup> indicating that AraC is a neurotoxin. For +AraC cultures, maximal stimulation/cell by Glu is doubled at 13 DIV compared to 6 DIV, with the same hyperbolic concentration-response curve. Cultures not treated with AraC exhibit greater maximal stimulation/cell by Glu at 13 DIV than at 6 DIV, but the response to Glu stimulation is gradual and ultimately equals the response of

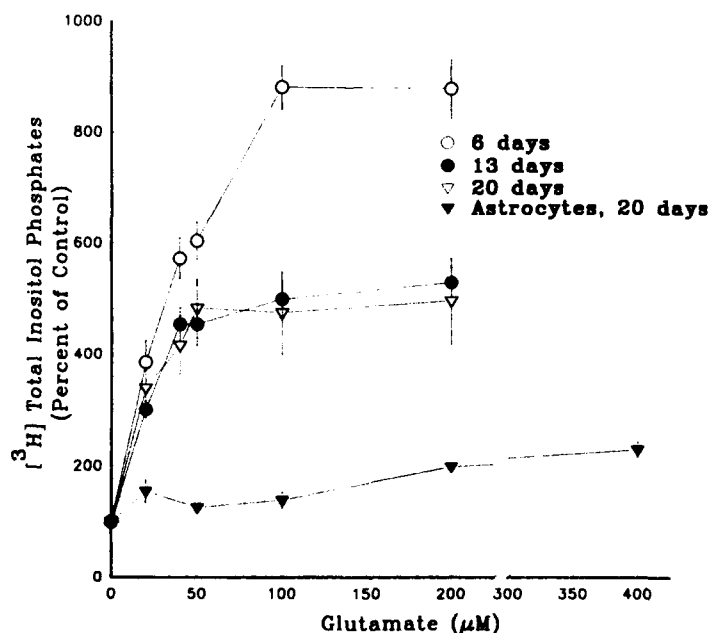


Fig. 1. Glutamate-stimulated total inositol phosphate accumulation in rat primary cortical cultures (+cytosine arabinoside) at 6 (open circles), 13 (filled circles) and 20 (open triangles) days *in vitro* and pure astrocyte cultures (filled triangles). Data are expressed as the mean percent of basal accumulation for each culture age/type  $\pm$  S.E.M. (8–16 determinations per data point).

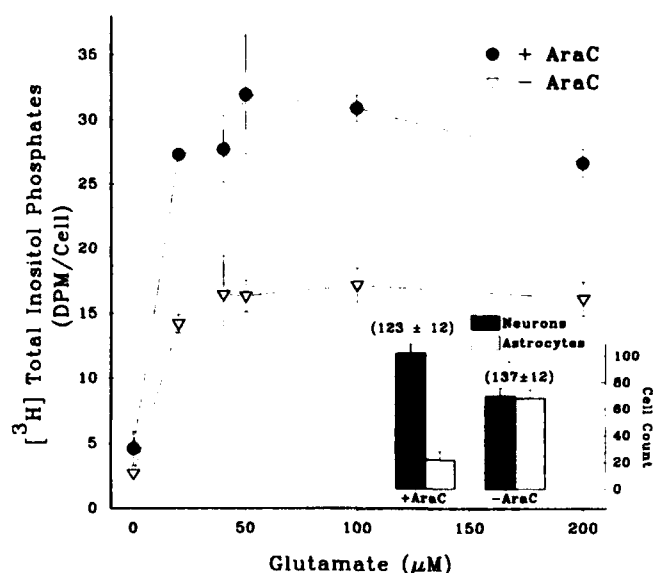


Fig. 2. Total inositol phosphate accumulation divided by total cell count in rat cortical cultures with and without cytosine arabinoside (AraC) treatment at 6 days *in vitro*. Data are expressed as mean  $\pm$  S.E.M. from a representative experiment. Inset depicts the number of neurons (filled bars) and astrocytes (open bars) present in the total cell count. Total cell count  $\pm$  S.E.M. is shown in parentheses above bars and was calculated as an average of counts from 6-8 wells on each plate.

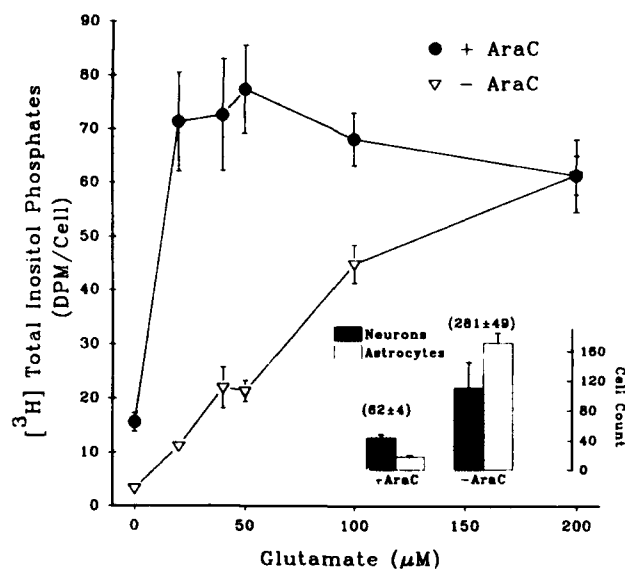


Fig. 3. Total inositol phosphate accumulation divided by total cell count in rat cortical cultures with and without cytosine arabinoside (AraC) treatment at 13 days *in vitro*. Data are expressed as mean  $\pm$  S.E.M. from a representative experiment. Inset depicts the number of neurons (filled bars) and astrocytes (open bars) present in the total cell count. Total cell count  $\pm$  S.E.M. is shown in parentheses above bars and was calculated as an average of counts from 6-8 wells on each plate.

+AraC cultures at the highest Glu concentration (200  $\mu$ M). Thus, in -AraC cultures at 13 DIV, the exponential increase in Glu-stimulated IP accumulation is not present as it was at 6 DIV.

Finally, at 20 DIV, total cell number remains similar to that at 13 DIV in +AraC and -AraC cultures (Fig. 4). Compared to previous weeks, the percentage of neurons remains similar in +AraC cultures while tending to decline in the -AraC cultures. Plateau levels of IP accumulation/cell for 20 DIV are virtually identical to 13 DIV for +AraC cultures, while the response/cell of -AraC cultures at this age continues to be depressed. The absence of an exponential component of the

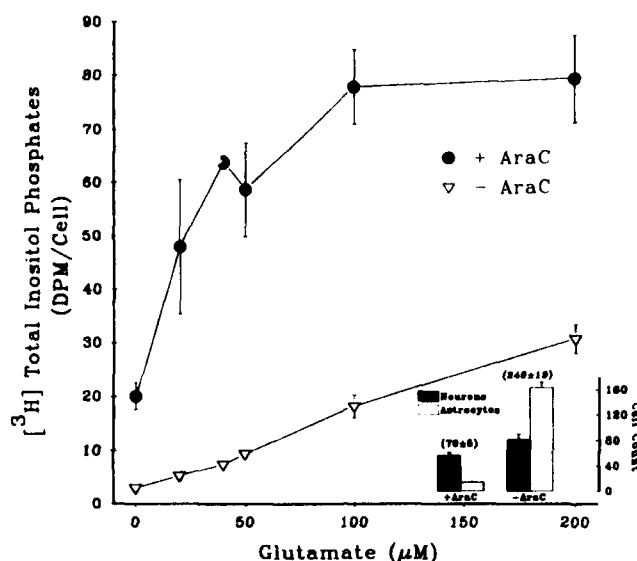


Fig. 4. Total inositol phosphate accumulation divided by total cell count in rat cortical cultures with and without cytosine arabinoside (AraC) treatment at 20 days *in vitro*. Data are expressed as mean  $\pm$  S.E.M. from a representative experiment. Inset depicts the number of neurons (filled bars) and astrocytes (open bars) present in the total cell count. Total cell count  $\pm$  S.E.M. is shown in parentheses above bars and was calculated as an average of counts from 6–8 wells on each plate.

concentration-response curve for  $-$ AraC cultures approximates that observed in pure astrocyte cultures at 20 DIV (Fig. 1). While basal levels of PI metabolism/cell for all weeks studied are similar in cultures not treated with AraC, the basal metabolism/cell increases progressively with DIV for cultures treated with AraC (Figs 2–4).

## DISCUSSION

In the present study, PI metabolism stimulated by Glu was determined to be concentration dependent, with decreasing  $EC_{50}$  values from 6 to 20 DIV. These data point to the fact that neuronal cultures (treated with AraC) demonstrate increased sensitivity to Glu with DIV. The effect of Glu on IP accumulation at 6 DIV is similar to that ( $EC_{50}=33 \mu M$ ) reported in other studies using rat cortical neurons.<sup>12</sup> Figure 1 also shows that maximal stimulation of PI metabolism by Glu decreases with DIV. This decreasing efficacy of Glu with DIV may be compared to the results of previous studies using cultured cerebellar granule cells.<sup>1</sup> However, the studies with cerebellar granule cells and the data in Fig. 1 do not include a measure of changing cell composition or number in developing mixed cultures. We therefore measured Glu-stimulated PI metabolism normalized to cell number (Figs 2–4) and found that, at least in cortical cultures, neuronal responsiveness/cell actually increases with increasing DIV.

Considering the proliferative potential of astrocytes, we addressed the contribution of astrocytes to the overall response of mixed cultures to Glu addition as a possible explanation for the developmental dynamics illustrated in Fig. 1. Figure 1 shows that pure astrocyte cultures are much less sensitive to Glu stimulation of PI metabolism than mixed neuronal cultures (similar to the response found by Pearce *et al.*<sup>14</sup>). We thus hypothesized that increasing numbers of astrocytes may account for the decreases in PI response after 6 DIV shown in Fig. 1.

Despite the ability of astrocytes to metabolize PI in response to Glu, we found that the major contribution of these cells in mixed cultures is one of Glu uptake. Given the similar cell number at 6 DIV with or without AraC treatment (Fig. 2), it is possible that the shift downward in the concentration-response curve for  $-$ AraC cultures is due to the larger number of astrocytes. Presumably, astrocyte uptake mechanisms deplete available concentrations of Glu for metabotropic receptor stimulation in neurons. In addition, there is a smaller percentage of the more sensitive cells, neurons, in the  $-$ AraC cultures. From 13 to 20 DIV in  $-$ AraC cultures (Figs 3 and

4), the reduction in responsiveness to Glu stimulation of PI metabolism may be due to an increasing percentage of astrocytes as well as maturation of Glu uptake capability.<sup>16</sup>

In contrast to the reduction of PI metabolism observed in cortical cultures with increasing astrocyte content, our studies indicate that neurons themselves become more sensitive to Glu-stimulated PI metabolism with increasing DIV. After normalizing PI metabolism to cell number, we found that in +AraC cultures, maximal response doubles from 6 DIV to 13 and 20 DIV (Figs 2–4). Since the percentage of neurons (approx. 80%) remains stable at these different ages, individual neuronal response is presumably increasing. Also, considering that the numbers of neurons at 6 and 20 DIV are similar in –AraC cultures (Figs 2 and 4), the increased maximal PI response/cell at 20 DIV may be explained by increased neuronal sensitivity to Glu at this age. Finally, it is of interest to note that while basal (unstimulated) PI metabolism/cell increases more than four-fold in neuron-enriched (+AraC) cultures from 6 to 20 DIV, basal PI metabolism remains stable in –AraC cultures during the same time *in vitro*.

In the present study, increases in mGluR1 responses may be indicative of synaptogenesis in culture in a manner analogous to the developmental dynamics of inositol phospholipid hydrolysis in synaptogenesis or synaptic plasticity *in vivo*.<sup>5,16</sup> Formation or maturation of these interneuronal connections may account for the increased basal inositol phospholipid metabolism demonstrated in neuronal cultures (+AraC) in this report (Figs 2–4). Since this increase in basal PI metabolism is not observed in –AraC cultures with increasing DIV, the release of endogenous stimulators of PI metabolism by communicating neurons may be blunted by the presence of astrocytes.

These considerations of synaptogenesis refer to intercellular mechanisms that may modulate basal PI hydrolysis, as well as response to stimulatory agents. Studies by Mundy *et al.*<sup>9</sup> provide a possible mechanism for intracellular modulation of PI hydrolysis throughout development. In their studies, where hippocampal and cortical receptor number did not change from young to aged rats, increases in receptor coupling to guanine nucleotide binding proteins or an increase in activation of phospholipase C were implicated to explain increases in PI metabolism. Another possibility is that the mGluR1 is increasing in number per neuron with DIV.

While our studies take into account changes in total cell number with DIV, both inter- and intracellular mechanisms may be responsible for the developmental changes seen in stimulated and unstimulated PI metabolism demonstrated in the present report. These multifold dynamic mechanisms may be acting independently or in concert.

It is evident from the data presented here that unbridled astrocyte proliferation suppresses the exponential concentration-dependent increase in Glu-stimulated PI metabolism by neurons in culture. Since pure astrocyte cultures demonstrate little PI response to Glu addition (at least at low concentrations of Glu), the major effect of these cells in mixed cultures appears to be a lowering of the effective concentration of Glu stimulating neuronal PI metabolism.

Developmental studies of mGluR1 sensitivity have traditionally used protein determinations to address total cellular material in brain slices.<sup>10,18</sup> Recently, Aronica *et al.*<sup>1</sup> have used DNA content to normalize developmental changes in IP formation measured in cerebellar granule cell cultures. In contrast, our cell culture studies used actual determinations of cell type and cell number. Consideration of these parameters revealed that neurons themselves become more sensitive to Glu stimulation of PI hydrolysis with increasing DIV. Continued study of cell cultures during development will need to address changing cell populations in the central nervous system. Similarly, studies utilizing more fully integrated systems such as the slice preparation may also need to evaluate the influence of astrocytes and other cell types on overall measured PI responses.

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